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Biosynthesis of silver nanoparticles using stem bark aqueous extracts of Pittosporum napaulense (DC.) Rehder& E.H Wilson. Their antibacterial activities.

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Abstract:

Plant mediated synthesis of nanoparticles has wide application in biomedicine due to its novel properties and its eco-friendly nature. The present study deals with the biosynthesis of silver nanoparticles (SNPs) from the stem bark aqueous extract of *Pittosporum napaulense*. The synthesized nanoparticles are characterized by the colour change, observed from gray to dark brown indicates the formation of nanoparticles and UV–VIS surface Plasmon resonance spectroscopy observed at 444 nm further confirmed the synthesized nanoparticles as SNPs. FTIR spectroscopic studies confirm that phenols, amines and halides of stem bark extract is mainly responsible for capping and stabilization of synthesized SNPs. The XRD data shows crystalline nature of nanoparticles and EDAX measurements reveals the presence of 37.95% Ag metal. Zeta potential at -29.7 mV the negative value indicates the high stability of nanoparticles. TEM microscopic analysis revealed that the size of synthesized SNPs ranging from 22.08 to 45.82 nm with spherical shape. Further, the antibacterial studies of synthesized SNPs show high activity towards *Staphylococcus aureus* with 35.50 mm diameter zone of inhibition followed by ,*Eschirichia coli* and *Bacillus subtilis*.

Keywords: Biomedicine, Plasmon Resonance, Capping, Crystalline, Zeta Potential, *Staphylococcus aureus*, Spherical.

INTRODUCTION:

Nanoparticles are being considered as cluster of atoms between 1 to 100 nm range Smaller the particle size has unique, chemical and physical properties and is very useful in biomedical science. Recent studies are focused towards synthesis of metals like, iron, copper, calcium, gold, palladium, zinc and silver nanoparticles using plant. Silver has been recognized its importance in chemistry, physics and biology due to its unique properties over the last few decades, synthesis and characterization of metal nanoparticles gained attention because of their peculiar properties compared to their bulk counterparts, having their high surface to volume ratio [1].Among the biological routes, plant and plant materials mediated nanoparticle synthesis is more advantageous than microbes and animal products. This is due to the presence of broad variability of biomolecules in plants that act as capping and reducing agents which in turn increase the rate of reduction and stabilization of silver nanoparticles [2]. Hence, among the metal nanoparticles, sliver nanoparticles (SNPs) synthesized from medicinal plants has received, much attention in various biological activities like antibacterial [3] and antifungal [4].The reducing agents involved in the synthesis include various water soluble metabolites such as alkaloids, phenolic compounds, terpenoids, flavones, quinines, organic acids, polysaccharides, proteins and co-enzymes which are available in the plant extract [5].Silver has been known to have strong broad spectrum antimicrobial activities even at low concentrations[6].

The selected medicinal plant *Pittosporum napaulense* (Pittosporaceae) (Fig.1) is called ‘Rakamuki’ (Telugu), ‘Kattusampangi’, Najundai’,‘Tammata’(Tamil), Tumari’, ‘Vikharl’ ‘Vekhali’(Marathi)**.**Theplant parts are used against skin diseases, piles and itches**.** Bark is aromatic, bitter and greenish black with resinous oil glands. In Ayurveda bark in high doses acts as narcotic used as antidote to snake poison, general weakness and also as a stimulant**.**

(A)

(B)

(C)

(D)

Fig.1 A. *Pittosporum napaulense.* B, C, D. Stem Bark

The narcotic action of the bark is due to the presence of yellow oleoresins, and also contains saponins and Pittosporins [7-11]. Bark is bitter and aromatic; possess narcotic properties used as febrifuge, chronic bronchitis which acts as good expectorant. Oil used for rheumatism, skin diseases, sprains, leprosy, bruises, sciatica, chest infections, opthalmia, cutaneous diseases, secondary syphilis and chronic rheumatism, supports the presence of glycosides[12]. In New Zealand Mori people used the gum, leaves, flowers and oils of *P. eugenoids*to anoint their bodies. Flowers, roots, bark and leaves are used as anti-inflammatory, antiseptic and in rheumatic disorders. Bark consists of oleoresins, triterpenoids, saponins, stigmasterols [13-16].

Material and Methods

Plant material collection and identification

Pittosporum napaulense was collected from Tirumala forest, during the months of July and December. The plant was authenticated by Prof. N.Yasodamma and voucher specimens AU 02, AU 03 were prepared as per the standard method [17] and deposited in the herbarium, Department of Botany.

Synthesis of SNPs

Dry powder 5 gms of the *P. napaulense* bark extracts with 100 ml of milli*q* water on boiling water bath for 1 hour. Filter the content with whatman No. 1 filter paper and stored at room temperature for green synthesis of SNPs. 5 ml of plant extract was taken in 250 ml conical flask, titrated with 50 ml of 1mM $Ag(NO_3)^2$ at 60-80°C with the help of magnetic stirrer. The contents were centrifuged at 10000 rpm for 20 minutes to avoid the presence of any biological impurities. Further, the synthesized nanoparticles were used for characterization and antimicrobial studies.[18]

Characterization of Silver Nanoparticles (SNPs)

UV–Vis absorption spectrum of *P.napaulense* bark extracts SNPs was measured by using Nanodrop 800. Zeta potential analysed by HORIBA SZ-100, Fourier-Transform Infra Red (FT-IR) spectra of synthesized SNPs were analyzed in the range of 4,000 to 500 cm-1 with an IRAFFINITY-1,IR by ATR method. Crystalline nature of metallic silver nanoparticles was examined using an X-ray diffractometer (XRD) from Bruker, D8 advance, Germany. XRD-6000 equipped with Cu Ka radiation source using Ni as filter at a setting of 40 kV/30 mA. Transmission electron microscopy (TEM) technique was used to visualize the morphology of the AgNps. The 200 kV ultra-high-resolution transmission electron microscope (FEI-TECNAI G2 20 TWIN).TEM Grid were prepared by placing a 5 µL AgNPs Solution on Carbon- Coated Copper grids and drying under lamp.

Antimicrobial studies of SNPs

The antimicrobial activity of green synthesized silver nanoparticles of *P.napaulense* stem bark extract was analyzed against two Gram positive bacterial strains like *Bacillus subtilis* (MTCC441), *Staphylococcus aureus* (MTCC731) *and* Two Gram negative bacterial strains like *Escherichia coli*,(MTCC443) and *Klebsiella pneumonia*(MTCC741) using Disc diffusion method [19]. Comparative studies were made with stem bark extract as a positive control, 1mM $Ag(NO_3)^2$ as negative control and *Streptomycin* as the standard. Sterile discs of 7mm size were prepared from whatman No.1 filter paper and 20 µl of each extract was loaded on separate discs with the help of micro pipette and allowed to air dry for one hour under aseptic conditions. Freshly prepared nutrient agar media for bacterial culture substrate was poured into sterile Petriplates and allowed 30 minutes for solidification. The plates were swabbed with microbial cultures and placed the previously prepared discs; the experiment was carried out in triplicates. The plates were incubated at 37 °C for 24 to 48 hrs then the diameter zone of inhibition was measured

Results

UV–visible spectral analysis:

The formation of Silver Nanoparticles of *P.napaulense* bark extracts was monitored by UV-VIS absorption spectra. The colour change from Grey to Dark Brown is observed and a typical absorption peak obtained at 444 nm, it is due to surface Plasmon resonance of silver nanoparticles in the reaction Mixture (fig.2 a,b).

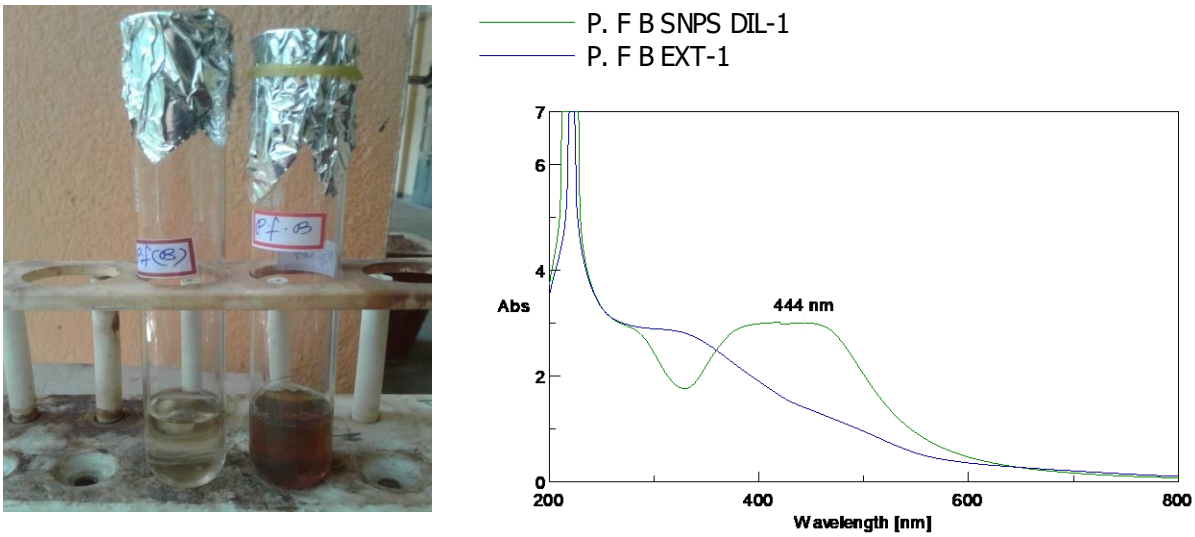


Fig.2 (a) Colourchangeof AgNPs of *P.napaulense* bark extracts grey to brown.(b)UV-VIS analysis of synthesized SNPs of *P.napaulense* bark extracts shows peak at 444 nm.

Fourier Transform infra-Red (FTIR) analysis:

FTIR spectrum of synthesized SNPs of *P.napaulense* bark extracts was carried out to know the possible biomolecules responsible for capping and stabilization of nanoparticles. For this the FTIR spectrum was analysed between the scan ranges from 4000 to 500 (fig.3).

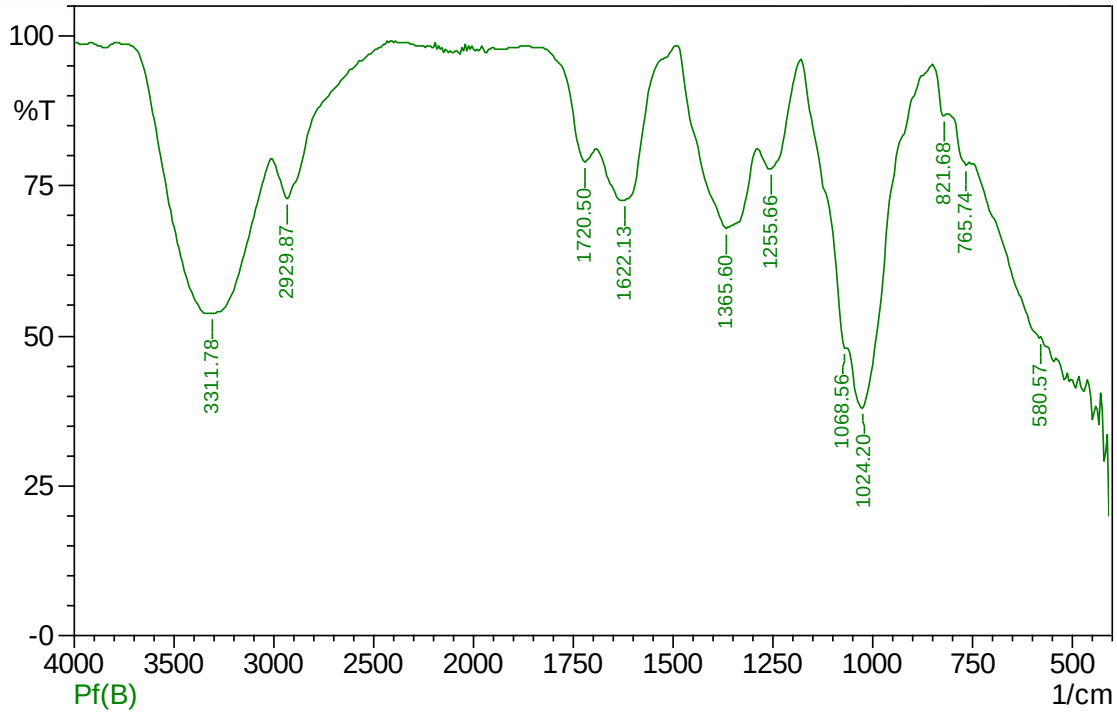


Fig.3 FTIR spectra of green synthesized SNPs from stem bark extract of ***P. napaulense***

3311.78 cm^{-1} assigned for O-H (Stretch) bond of phenols; 2929.87 cm^{-1} for C-H alkanes;1720.50 cm^{-1} for C-H saturated aliphatic; 1622.13 cm^{-1} for C-C Amines; 1365.60 cm^{-1} for N-O (Symmetric Stretch) Nitro compound; 1255.66 $cm^{-1;}$ for C-N aromatic amines; 1068.56 $cm^{-1}$for C-N aliphatic amines ; 580.57 cm^{-1} for C-

Br alkyl halides .These FTIR studies suggested that the hydroxyl groups of phenols and amide groups of proteins forming a layer to the nanoparticles and acting as capping agents to prevent agglomeration and providing stability to the medium.

Particle size and Zeta potential analysis:

The particle size of *P.napaulense* bark extracts AgNPs is detected by the intensity and laser diffraction method using the biosynthesized colloidal solution in which the AgNPs are polydispersed in mixture solution. The distribution of AgNPs found 4.1nm with an average size. (Fig.4 a&b). And PI value 0.408 (poly disperse index). Further the zeta potential analysis of AgNPs was detected to be 29.7 mV, due to its high negative zeta potential it prevent the AgNPs from agglomeration in the medium, leading to long term stability, because of the electrostatic repulsive force between the AgNPs. Zeta potential is an essential parameter for the characterization of stability in aqueous nanosuspensions minimum of ± 30 mV Zeta potential values is required for indication of stable nanosuspension.[20]. Zeta potential at-29.7mV, negative value indicates the high stability of Nanoparticles. So, these results clearly indicated that the particles are fairly stable due to the electrostatic repulsion

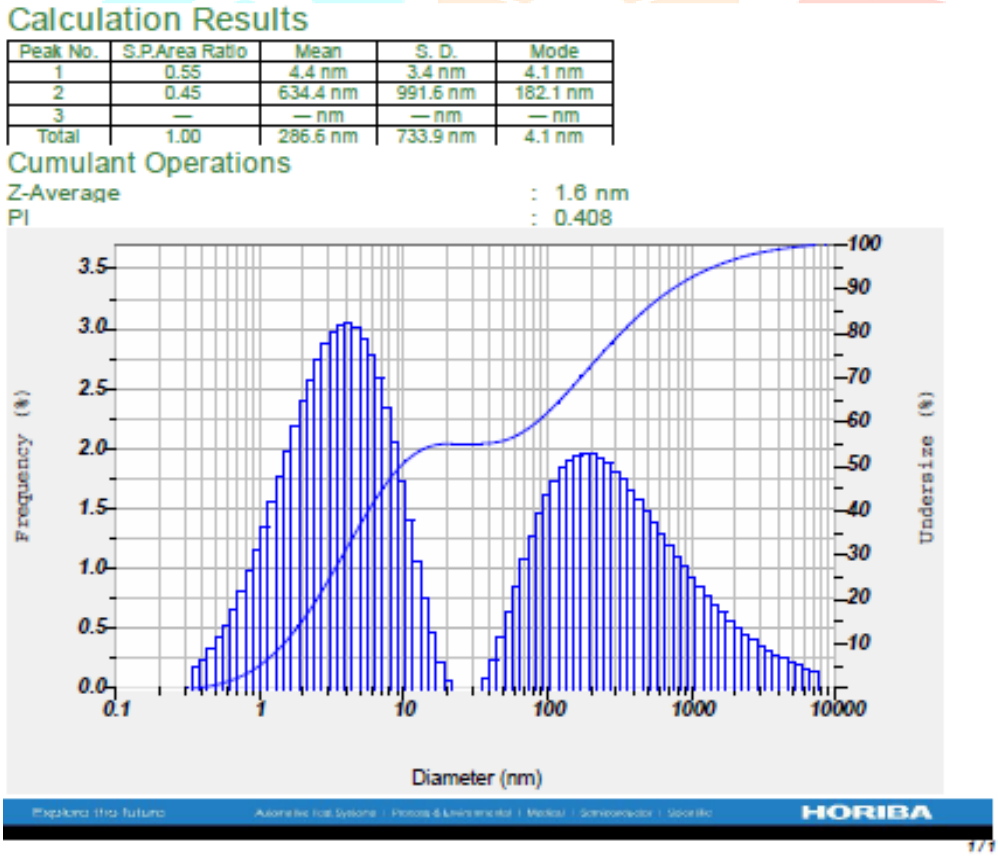


Fig.4 (a) Particle size of green synthesized SNPs from stem Bark of *Pittosporum napaulense.*

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-29.7 mV	-0.000231 cm2/Vs
2	— mV	— cm2/Vs
3	— mV	— cm2/Vs

Zeta Potential (Mean) : -29.7 mV
Electrophoretic Mobility Mean : -0.000231 cm2/Vs

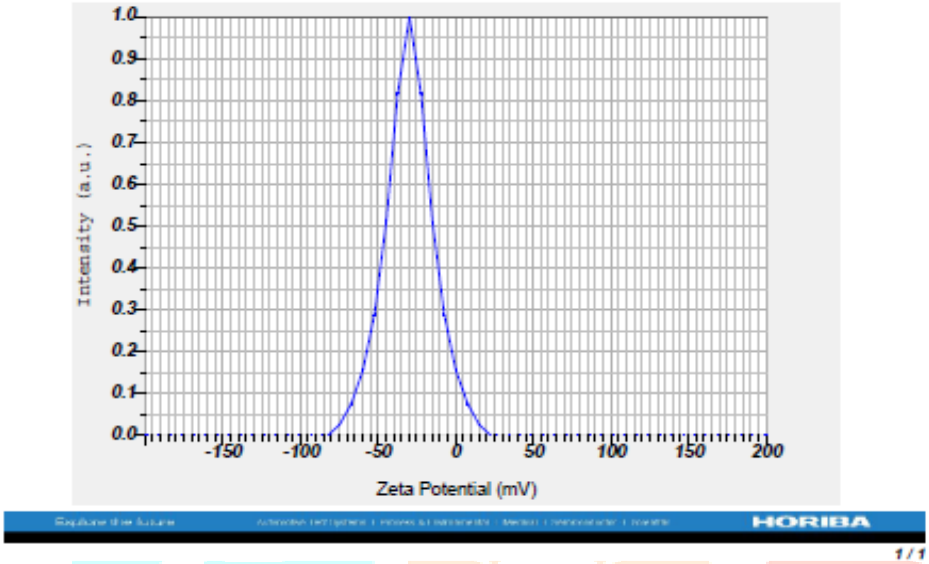


Fig.4 (b) Zeta potential of green synthesized SNPs from stem Bark of *Pittosporum napaulense.*

XRD Analysis:

The nature of the nanoparticles synthesized from bark extract was analysed by X-ray diffraction analysis. The XRD Shows (fig.5) *P.napaulense* derived SNPs. with an intensive peak at 27.29 31.86 37.64 43.84 45.86 54.46 57.02 64.15 77.06 of 2θ degrees of X-axis correspond to 110, 120, 111, 200, 131, 202, 220, 220 and 311 Bragg Reflections of Y-axis (JCPDDS No: 89-3722 &841261). These Bragg reflections confirm that the nanoparticles are crystalline in nature.

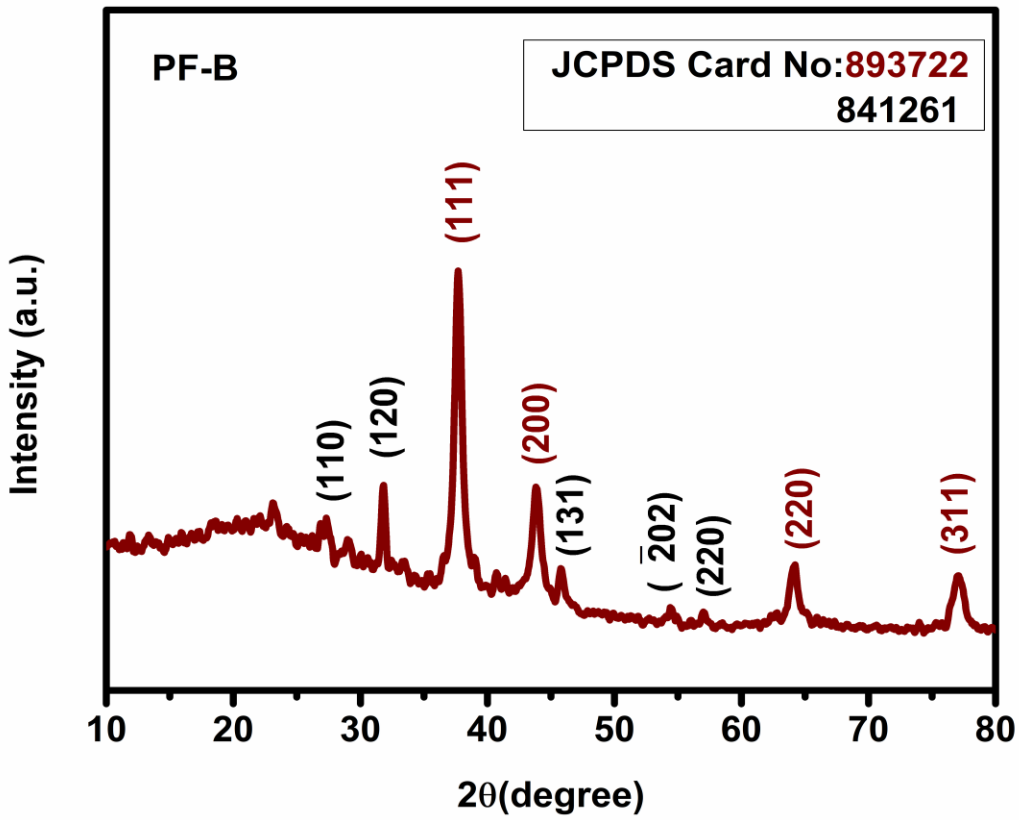


Fig 5 XRD pattern of green synthesized SNPs from bark extract of *Pittosporum napaulense*.

Energy Dispersive X-ray (EDAX) Analysis:

TEM with EDAX analysis (fig 6) provides further insight into the morphology and size of the nanoparticles along with presence of different metal concentrations in the sample. EDAX analysis was performed to know the percentage of Ag present in the *P.napaulense* AgNps sample. The EDAX spectra shows strong silver 37.95 % absorption peak along with different elements with their weight percentage like Carbon 43.12 %, Copper 16.58%, oxygen 1.40%, and Sulfur 0.95% and the results indicated that the reaction product has high purity of SNPs .Presence of C, Cu, O and S in the sample analyzed by EDAX indicates proteins as a capping material towards these silver nanoparticles.

Transmission Electron Microscopy (TEM): Higher resolution studies with TEM analysis, to know the size, morphology and agglomeration pattern of nanoparticles.100 nm resolution studies of nanoparticles *P.napaulense* reveals the nanoparticles are 22.08-45.82 nm in size owing spherical shape without any agglomeration observed between the particles

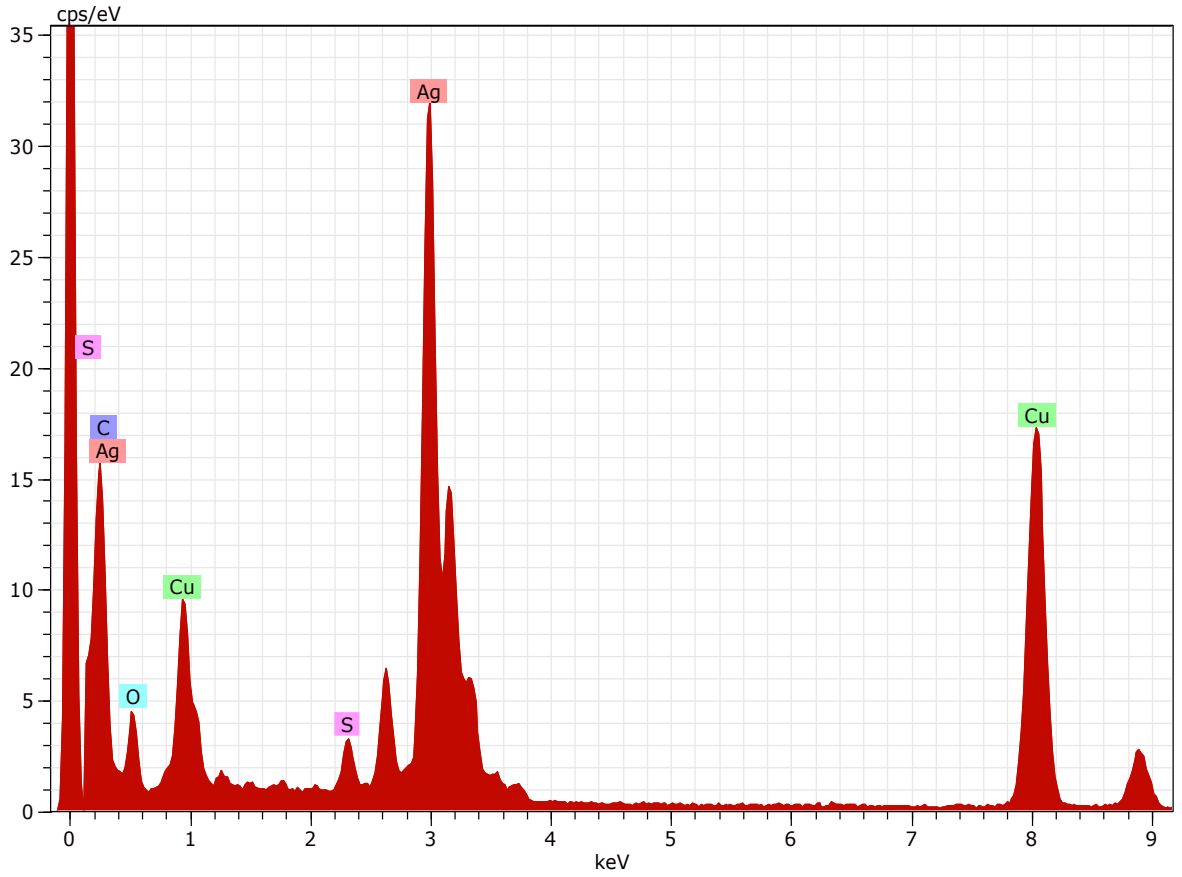
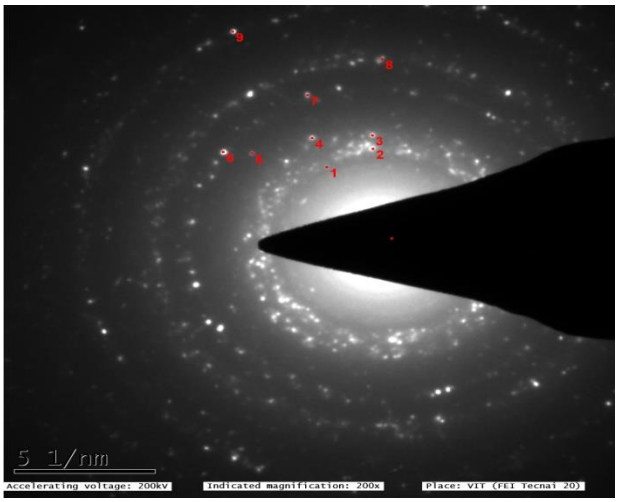


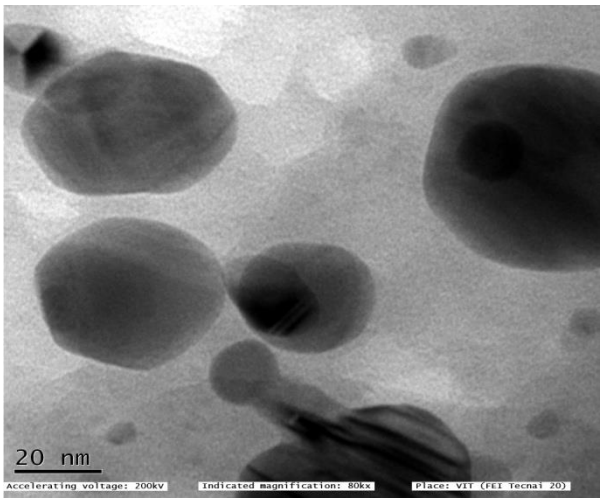
Fig 6EDX analysis of green Synthesized **AgNPs** of ***P.napualense***

Spectrum: Spectrum 1492-Pf-B

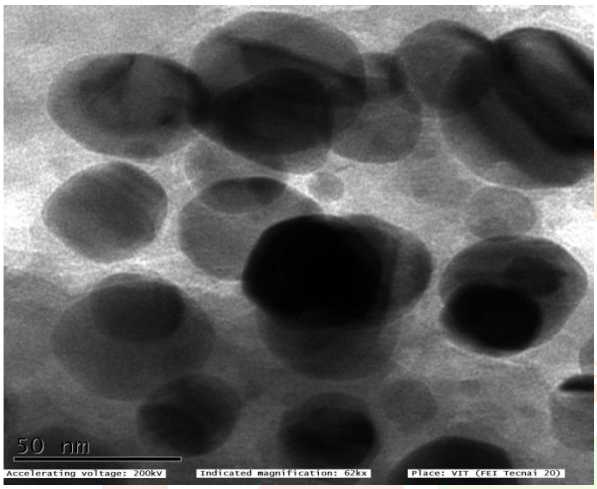
Element	Series	Net unn.	C norm. [wt.%]	C Atom. [wt.%]	C Error [at.%]	(3 Sigma) [wt.%]
Silver	K-series	7348	37.95	37.95	8.14	3.79
Copper	K-series	37765	16.58	16.58	6.04	1.59
Carbon	K-series	12515	43.12	43.12	83.11	4.12
Oxygen	K-series	2841	1.40	1.40	2.02	0.22
Sulfur	K-series	3513	0.95	0.95	0.69	0.17
		Total:	100.00	100.00	100.00	



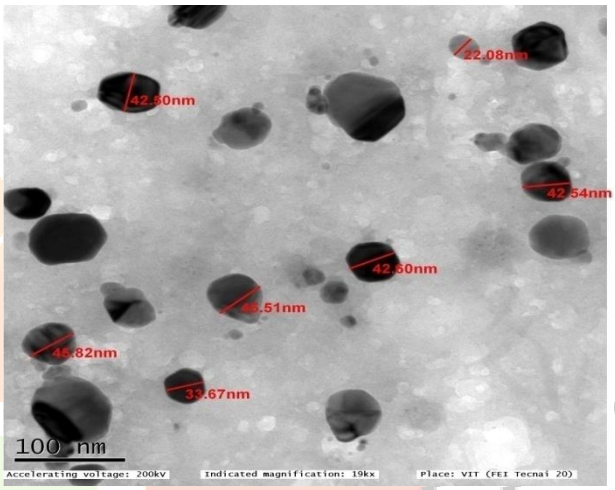
(A)



(B)



(C)



(D)

Fig 7(A) Selected area electron diffraction (SAED) of green synthesized *P.napaulense* SNPs, (B) 20 nm resolution SNPs. shows mostly spherical shaped (C) 50 nm resolution nanoparticles with spherical (D) 100 nm resolution the size of the nano particles between 22.08-45.82 nm shows mostly spherical shaped.

Antimicrobial Activity of AgNPs:

These green synthesized silver nanoparticles of *P.napaulense* were assessed for antimicrobial activities against two gram positive and Two gram negative bacteria, the highest inhibition zones were observed against *Staphylococcus aureus*35.50 mm followed *Escherichia coli* 33.75 mm (Fig 8, 9 and Table 1).

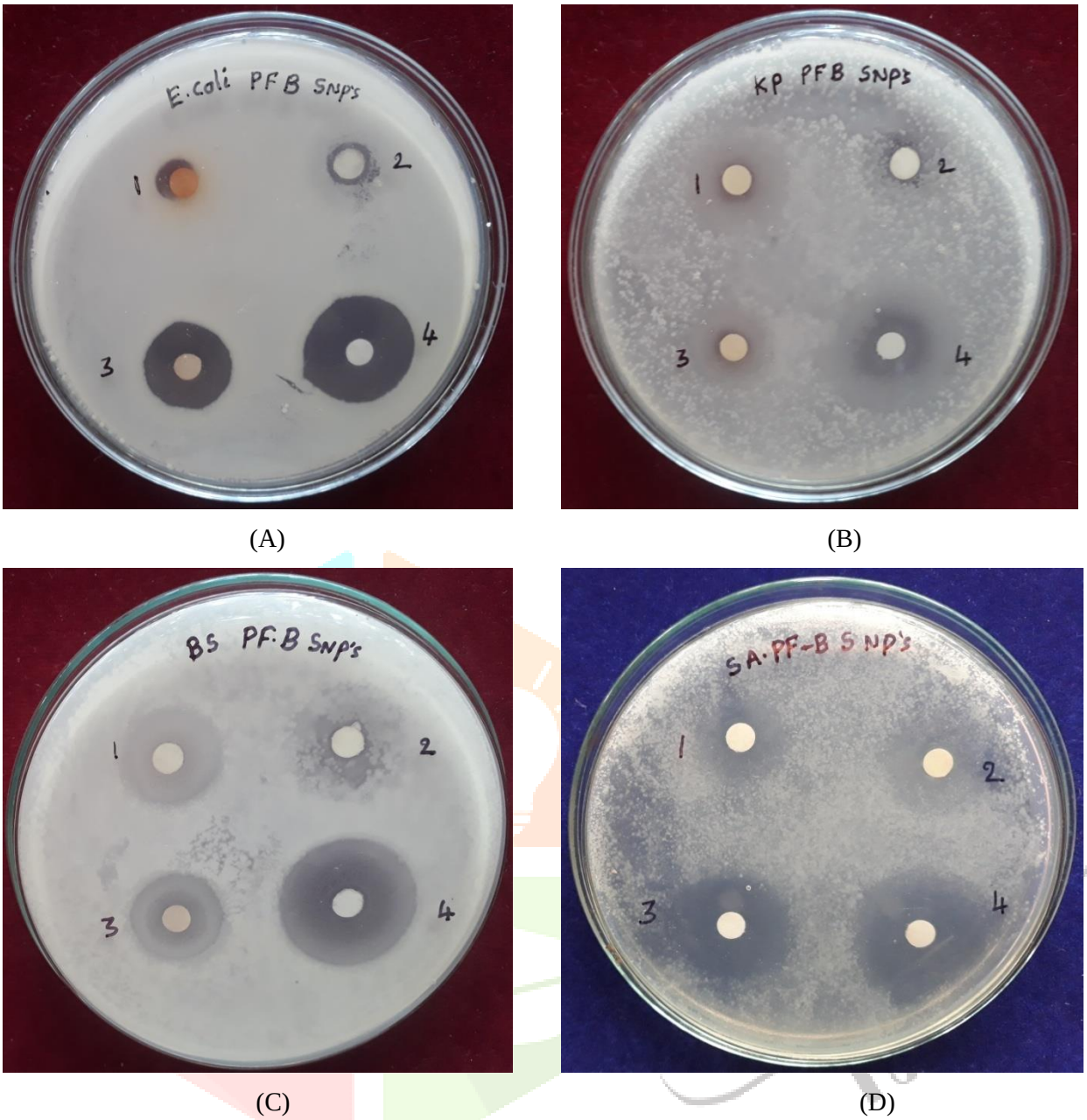


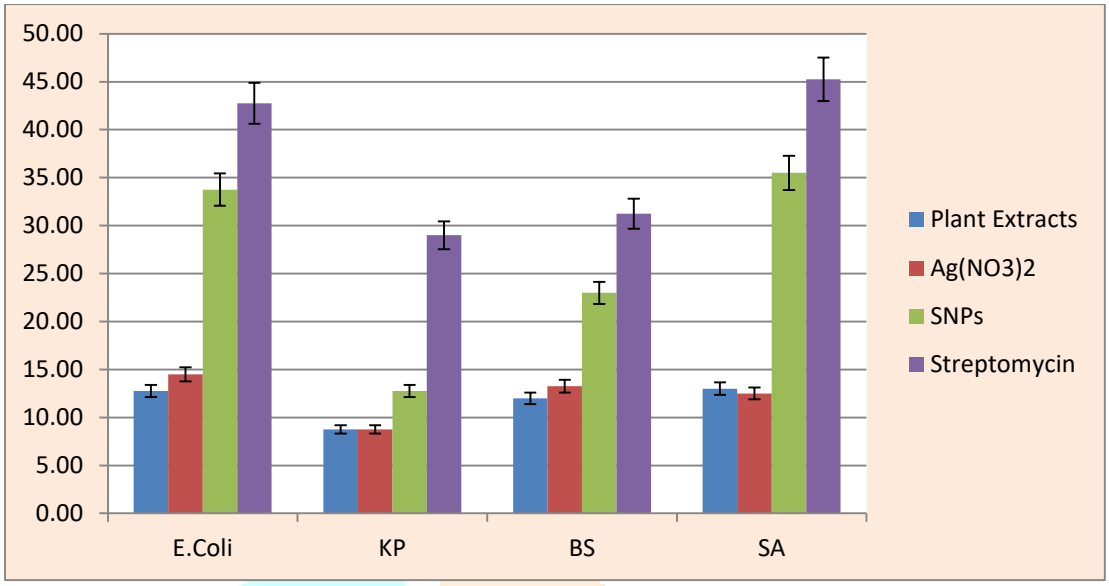
Fig 8 (a) *Escherichia coli*, **(b)** *Klebsiella pneumonia*, **(c)** *Bacillus subtilis* and **(d)** *Staphylococcus aureus* **Note: 1)** Aqueous Extract **2)** Ag(NO3)2 **3)** AgNPs *P..napaulense***4)** *Streptomycin*

Table 1 Effect of different extracts and green synthesized silver nanoparticles of *P.napaulense* bark extracts on bacterial Strains

Organism	Bark Extracts	$Ag(NO_3)_2$	*P.napaulense* SNPs	*Streptomycin*
E.Coli	12.75 ± 0.48	14.5 ± 0.29	33.75 ± 0.25	42.75 ± 0.48
KP	8.75 ± 0.25	8.75 ± 0.48	12.75 ± 0.25	29 ± 0.41
BS	12 ± 0.41	13.25 ± 0.25	23 ± 0.41	31.25 ± 0.48
SA	13 ± 0.41	12.50 ± 0.29	35.50 ± 0.29	45.25 ± 0.48

E.coli: *Escherichia coli*, **KP:** *Klebsiella pneumonia*, **BS:** *Bacillus subtilis* and **SA:** *Staphylococcus aureus*

All the data are expressed as **mean ± SEM:** ***p<0.01, **p<0.02, *p<0.03 as compared to Control group, n=4; (One –Way ANOVA followed by Dunnett's test).



E.coli: *Escherichia coli*, **KP:** *Klebsiella pneumonia*, **BS:** *Bacillus subtilis* and **SA:** *Staphylococcus aureus*

Fig 9 Zone of inhibition of different extracts and AgNPs of ***P.napaulense*** on bacterial strains.

The SNPs shows less significant effect on Gram negative bacteria. the SNPs penetrate inside the bacteria and fungi causing damage by interacting with electrons of phosphorous and sulphur containing Compounds such as DNA and proteins, resulting in cell Death [21].

Discussion

Leaf oil of *P. senacia* contains sesquiterpenes δ- cadinene 11.3% α murolol 15.9% and α- cadinol 19.0% **[22]**. *P. viridiflorum* leaf oilcontains sesquiterpene δ-cadinene 10.6% and α-cadinol 18.3%. The major fruit oils consistssabinene 13.2%, decanal 10.3% β- elemene 9.5%, β-pinene 8.7%, α-pinene 8.0% and α-cadinol 8.1%. The leaf and fruit oils had similar inhibitory effects on all bacterial strains except fruit oil with less activity against *Pseudomonas aeruginosa*, **[23]**. The leaves of *P. viridiflorum* consists of 15 components and yield 85.4% of oils where as the mature fruits contains 26 components andyield 94.5% of oils which showed effective antimicrobial activity against gram negative bacterial strains *S. aureus* and *Salmonella typhi*. The leaves and fruits of *P. neilgherrensis* contain 21 components of 97.6%of oils; fruits consists 20 components of 81.3% of oils. Undecane 62.2% as the major component in the leaf followed by caryophyllene oxide 9.0%, β-caryophyllene 8.7%. β- Selinene 11.9%, fruit oil consists of Undecane 11.3%, nonane 8.8% and α-pinene 8.4%. Oils show moderate activity against most of the tested gram-positive and gram-negative bacteria **[24]**. Essential oils from the bark of *P. dasycaulon* consists of dodencanal 53.43%, undecane 20.84%, hexadecanal 9.95% dodecanoic acid 3.6 and 1-tridecanol 2.15%. These oils also shows effective antimicrobial activity against all

gram positive and gram negative bacteria except on *Bacillus subtilis*. With the Minimum Inhibitory Concentration ranges from 25-100 µl/ml **[25].**

P. undulatum contain monoterpinoids, diterpionoids, sesquiterpinoids and alkanes, showed effective antimicrobial activity against *P.aureus, S. epidermis* and *S.aeruginosa* **[26].**Essential oils antifungal activity against *A.flavus* found the inhibition of the aflotoxin B1 production **[27]**. Crude leaf saponin mixture of *P.tetrasporum* leaves showed effective antifungal activity with 13.3mm Dz to that of the *Nystatin*12mm Dz **[28]**.Water, Ethyl acetate and chloroform extracts of *P. phylliraeoides* with major phytoconstituents as alkaloids, flavonoids, phenols, saponins and proanthocyanidins, shows effective antimicrobial against 14 bacterial strains and 1fungal strain. But no activity against *Candida albicans.***[29-30]**.phytochemical screening, antibacterial and antifungal studies of *Pittosporum floribundum (P.napaulense)* supports the herbal and traditional uses against skin diseases, arthritis, inflammatory, spasmodic, sciatica, sprains, bronchitis, chest pains, antidote to snake bite, narcotic and also in curing leprosy may be due to the presence of the major secondary metabolites in the crude extracts and the effective activity with lowest concentrations on all bacterial and fungal strains which may cause the health disorders to that of the herbal uses of *Pittosporum floribundum.* antimicrobial activity of bark methanol extracts exhibits effective inhibition with 16-25mm of zone of inhibition [31**]**.The synthesized AgNPs of *Pittosporum senacia leaf* showed excellent antimicrobial activity against *Escherichia coli* using both chemically and biologically synthesized silver nanoparticles, with inhibition zone of 20 mm and 21.67 mm respectively.[32].

Conclusions

The biosynthesized silver nanoparticles using *Pittosporum napauelnse* Stem bark extract proved excellent antimicrobial activity against *Staphylococcus aureus* with 35.50 mm diameter zone of inhibition Hence the biological approach appear to be cost efficient alternate to conventional physical and chemical method of silver nanoparticles synthesis and would be suitable for developing a biological process for large scale production .

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